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CYTOTOXIC AND APOPTOSIS-INDUCING EFFECT OF LUTEOLIN ISOLATED FROM *FERONIA LIMONIA* ON HEPG2 CELLS

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most commonly detected cancer. Traditional medicines have long been used for the treatment of this cancer. The objective of this study was to screen the anticancer activities in human hepatocellular carcinoma (HepG2) cell line of the isolated luteolin from *Feronia limonia.* Cytotoxic and apoptotic activities of luteolin were performed on HepG2 cell line by MTT assay and ethidium bromide (EtBr) staining & DNA fragmentation technique respectively. Vincristine was used as the standard drug. The results showed that the luteolin at 100 µg/ml has the highest cytotoxic activity with the IC_{50} (concentration that inhibits cell growth by 50%) value of 100 µg/ml with 48.23 % inhibition. The compound exhibited the highest apoptotic induction at the IC50 concentration. This study has confirmed the potential anti-cancer activity of luteolin from *Feronia limonia* which can be further developed as a novel drug.

Key words: Hepatocellular carcinoma, HepG2, MTT assay, *Feronia Limonia.*

INTRODUCTION

Cancer is the severe chronic disease that is found worldwide with increasingly high rate of morbidity and mortality (Siege et al., 2012). Therefore, it is of interest to search for cancer preventive agent from natural source. One preferable pharmaco-dynamic endpoint for cancer treatment is via inducing apoptotic cell death. Hence, apoptosis induction is the primary goal of chemotherapy. Apoptosis is the cell mechanism that balances between cancer cell proliferation and damage irreparable cell including DNA damage. Then, dead cells are phagocytosed by macrophages. The advantage of this death mode does not lead to inflammation in neighboring cells similar to necrosis (Brunelle and Zhang, 2010).

Naturally derived drugs are already proven as very effective in preventing hazardous effects generated by the chemicals during the chemotherapeutic treatment of various cancers (Khosit et al., 2008). These drugs have been attributed to their ability to target the mechanism of cancer cell division (Shaik et al., 2011). The mechanism underlying their pharmacological activities is though not clear; it has been speculated for their potentiality to inhibit certain enzymes, quenching of free radical generation or modulation of steroid hormone concentrations. Utilization of pharmaceutical compounds from plants in treatment of diseases has been of specific importance. Isolation and identification of some potent anticancer compounds, such as colchicine and taxol, as natural anticancer compounds, has encouraged scientists to screen different parts of plant species against cancer cell lines (Huang et al.,1986; Shokrzadeh & Saeedi,

2010). The aim of the present study was to evaluate the cytotoxic and apoptotic activities of luteolin in HepG2 cell line.

MATERIALS AND METHODS

Plant material:
Luteolin from fruit pulp of Feronia limonia was isolated according to our previous report (Jayashree & Ramesh, 2014) and used for the present anticancer activity.

Cell Culture:
Human hepatocellular carcinoma (HepG2) cell lines were cultured in DMEM (2mM L-glutamine, 100 g/ml of streptomycin, 100 U/ml of penicillin) medium supplemented with 10% fetal bovine serum and maintained in a 5% CO2 humidified incubator at 37°C. Cells were seeded at a density of 1×10^5 cells/ml.

Cytotoxic assay:

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay :
MTT assay was performed to assess the cytotoxicity of the plant extracts. (MTT is a yellow dye, which is reduced into purple formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells). HepG2 cells were cultured in 96-well microtiter plates and were treated with varying concentrations of the compound (50 µg/ml and 100 µg/ml) for 48 hrs. At the end of treatment period, to each well, 20 µl of MTT was added. After addition of MTT, the plates were incubated for 3 h in a dark chamber. Then, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm using microplate reader (Mosmann, 1983).

The percentage of cytotoxicity compared to the untreated cells was determined by the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated cell}}{\text{OD of control cell}} \times 100$$

The results were generated from three independent experiments; each experiment was performed in triplicate. The IC_{50} values were calculated.

Apoptosis assay:
Fluorescence based apoptosis was determined by using ethidium bromide (EtBr) staining method. Cells (approximately 1 x 10^6/ml) were seeded in 24-well plate along with 100µg/ml of plant extract and then incubated at 37^0C for different time period (0 to 6 hrs.). The cells are washed separately with PBS and treated with EtBr (100 µg/ml), observed under fluorescent microscopy using a blue filter, and photographed. Viable cells appear green in color.

DNA Fragmentation:
For the DNA fragmentation assay (Boon etal., 2007), 0.5 ml of the cell suspension was centrifuged and the pellet was collected, dissolved in TE (Tris-EDTA) buffer with vigorous shaking. The mixture was centrifuged and the supernatant was transferred carefully to separate new tubes. To the pellet, 0.5 ml of TE buffer was added, 0.5 ml of Ice-cold 1M NaCl and 0.7 ml of ice-cold isopropanol. The mixture was incubated overnight at -20°C to enhance precipitation. The content was centrifuged further to obtain pellet and was purified by repeated wash with 0.5 ml of 70% ice cold ethanol. The final pellet obtained was air dried and dissolved in 20-50µl of TE (Tris-EDTA) buffer. The fragmentation patterns of HEpG-2 cell lines were analyzed and compared with their control.

RESULTS AND DISCUSSION

Plant origin compounds are promising source of anti-infective and anticancer chemotherapeutic agents. Our study describes the potential use of *Feronia limonia* as a source of anti-cancer drug. The isolated compound luteolin was tested for its cytotoxic and apoptotic properties against the hepatocellular carcinoma (HepG2) cells *in vitro*. A dose of 50µg/ml and 100µg/ml was effective in inducing cytotoxicity in the cancer cells. Effective reduction in the viability of cancer cells were seen as determined by the MTT assay. The luteolin also induced apoptosis in the cancer cells as proved by the staining techniques

showing typical apoptotic features and a synergistic anticancer protective effect of the compound.

Studies on the isolated compound luteolin has shown a significant cytotoxicity against HepG2 liver cancer cell line in a dose dependant manner. When the isolated compound was added at different concentrations (50 and 100 µg/ml) to cultured HepG2 cells, and incubated for 48 hrs, it was observed that the compound was cytotoxic to the liver cancer cell line at all the tested concentrations (Table 1).

Table 1: MTT cytotoxicity assay

Conc. of isolated luteolin (µg/ml)	OD @ 540 nm	% viability	% inhibition
Control	0.815	100	0
50	0.670	82.20	17.80
100	0.422	51.77	48.23
vincristine	0.390	47.85	52.15

Values represent mean ± SD of three replicates

The cytotoxicity of isolated compound luteolin was equal to that of vincristine, a well known standard anticancer agent. Cell count and percentage viability of HepG2 cells treated with compound clearly indicate that it has inhibited the proliferation of HepG2 cells at 50and 100 µg/ml concentrations (percentage viability decreased as the concentration of compound increased and percentage inhibition increased as the concentration of compound increased) when compared to that of the controls. The maximum anticancer activity (48.23% inhibition) was recorded in the cell line culture supplemented with 100 µg/ml. On the other hand percentage of Viability was decreased from 82.2% to 51.77% while compound doses were increased from 50 to 100 µg/ml respectively. IC_{50} value was calculated to be around 100µg/ml of luteolin. This study provides evidence that isolated luteolin acts as a cytotoxic agent in cancer cell lines. In cell culture model, luteolin markedly reduced viable cancer cell count and caused cell death. This was supported by observed cytotoxicity in HepG2 cells. Results from our study demonstrate that IC_{50} of isolated luteolin on cancer cell line was lower than normal cell line. The higher IC_{50} of luteolin on normal cells compared with cancer cells can be resulted from dysfunction of cellular organisms following cancer incidence, which cause higher rate of proliferation and increased cellular intake. Also, disorders in defensive systems of cancer cells and effusion insufficiency to escape toxic substances, can lead to inhibition of the growth of cancer cells in comparison with normal cells, via lower amounts of cytotoxic compounds (Hultberg et al., 1999; Van et al., 2001). This observation of our study was in correlation with the results of previous studies conducted by Islam et al (Islam, 2009). Similar studies indicate that different phytochemicals causes cell damage in different ways by treatment of alkaloid isolated from *Catharanthus roseus* which binds to the microtubule and prevents mitotic cell proliferation (Sakarkar & Deshmukh, 2011). Study also shows that the flavonoids, tannins and other phytochemicals produce cytotoxic effect on tumor cells line (Dhanamani et al.,2011). It is also suggested that herbal drugs play anticancer role by enhancing immune system as well as detoxifying body, inhibition of angiogenesis and also cell differentiation (Prakash et al., 2011; Yu et al., 2013).

The key feature of a potential antitumor drug has the apoptosis-induction capacity rather than the necrosis induction. Cancer cells treated with any drug will show morphological features of apoptosis that includes cell shrinkage, membrane blebbing, nuclear condensation and apoptotic body formation (Ema et al., 2013). Results of the present study showed that the morphological changes were typical of apoptosis indicating the anticancer activities of the compound luteolin. Luteolin was able to induce apoptosis in HepG2 cells after 48 h incubation. The most active apoptosis induction was at 100µg/ml concentration observed as morphological changes to the nuclei in the dead cells: chromatin condensation, nuclear-fragmentation, and appearance of apoptotic bodies (Figure 1-4). DNA fragmentation assay is similar to vincristine which is used as positive control; treatment with the luteolin at 50 and 100 µg/ml

also induced DNA ladder formation (Figure 5). Together, these results confirmed that the ethanolic luteolin at both the doses induced apoptosis in human hepatic cancer cells.

Figures 1 to 5. Luteolin induced apoptosis in HepG2 cells

Figure 1: Control plate with normal HepG2 cells

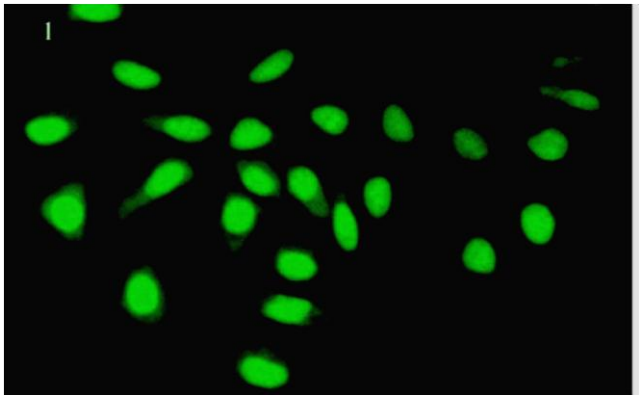


Figure 2: HepG2 treated with 50µg/ml of isolated Luteolin

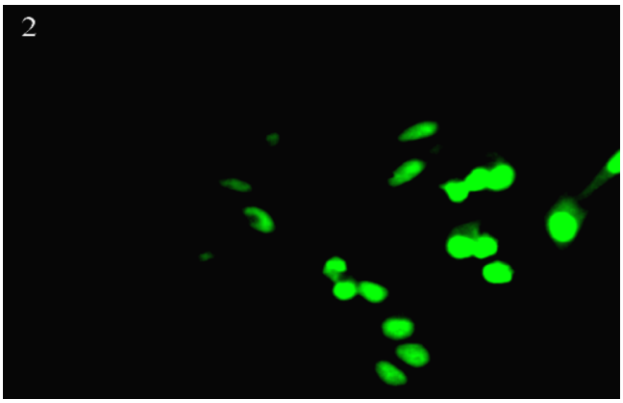


Figure 3: HepG2 cells treated with 100µg/ml of isolated Luteolin

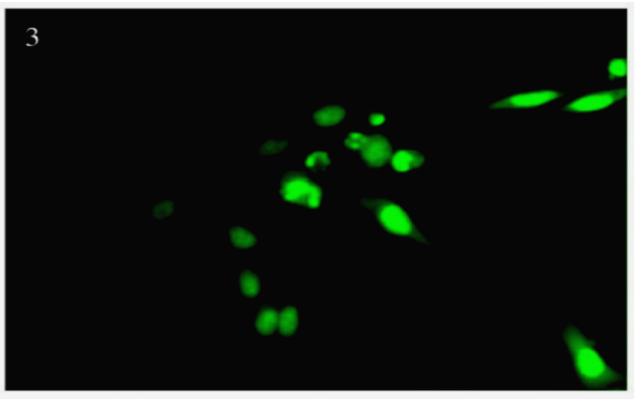


Figure 4: HepG2 cells treated with 10µg/ml of vincristine

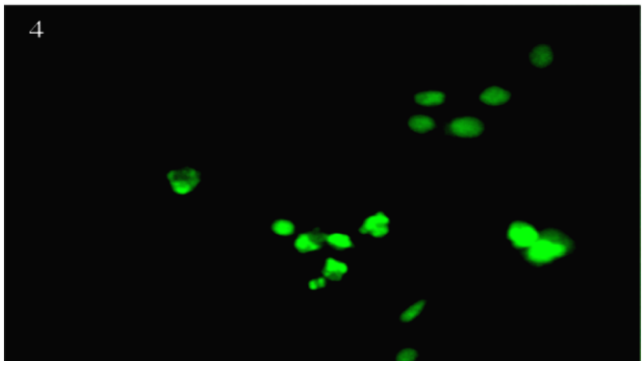
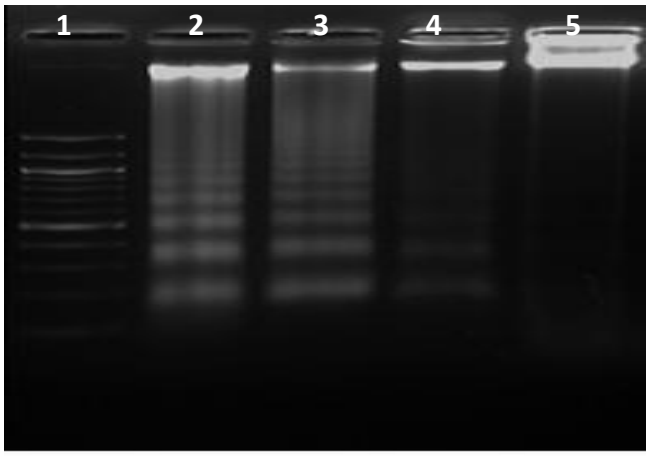


Figure 5: DNA fragmentation assay for the detection of apoptosis (Lane 1: DNA marker; Lane 2: 10 µg/ml Vincristine; Lane 3: 100 µg/ml isolated luteolin; Lane 4: 50 µg/ml isolated luteolin and Lane 5: Control).



The study is supported by previous studies on HepG2 cell lines by many plants. Methanolic extract of *Grewia hirsute* possesses a significant antioxidant and anti-proliferative potential when tested against HepG2 cell lines (Chang et al., 2002). *Philodendron selloum* and *Terminalia bellerica* plant methanol extracts treated to HepG2 showed hepatocyte denegenration, decrease in the number of cancer cells and necrotic debris as observed in light microscope (Prayong et al., 2008). Ethanolic extract of *Piper sarmentosum* was shown to trigger cell death in HepG2 through apoptosis, through morphological analysis using Giemsa and AO/EtBr staining procedures (Al-Rashidi et al., 2011) which is on par with the results obtained in our study. Thus, our results show that the

isolated compound luteolin from fruit pulp of *Feronia limonia* has a potential effect as anticancer chemotherapeutic agent well supported by the cytotoxic and apoptotic properties against the HepG2 cells *in vitro* to augment its effect.

CONCLUSION

Although several new approaches are coming up for drug discovery in recent years, none of them could replace the natural products derived from plant and animal origin [20]. Wholesome consumption of plant materials in traditional food habit amongst different tribes may provide important clue to the discovery of potent plant principles for combating the dreaded diseases like cancer. The findings of the present study are encouraging for nutraceutical and pharmacological evaluation of the plant. It also proves the potential use of the traditional medicinal plant *Feronia limonia* in the treatment of liver cancers. Study demonstrated the tendency to inhibit proliferation and induce apoptosis, respectively in human hepatocellular carcinoma cell line indicating the possibility for the development of anti-cancer drug.

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